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Iron-Responsive Regulation of Biofilm Formation in *Staphylococcus aureus* Involves Fur-Dependent and Fur-Independent Mechanisms

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We have shown that *Staphylococcus aureus* biofilm production is induced in iron-restricted conditions and is repressed by iron via a Fur-independent mechanism, while Fur has both positive and negative regulatory roles in low iron. Furthermore, there is no significant increase in polymeric *N*-acetylglucosamine polysaccharide expression to account for induction of biofilms in low iron.

Staphylococcus aureus is one of the most frequently isolated pathogens associated with nosocomial sepsis. Patients particularly at risk of developing staphylococcal infections are those with indwelling medical devices, which *S. aureus* colonizes as a biofilm (11). Several environmental factors have been shown to influence *S. aureus* biofilm formation, including anaerobic growth (5), osmotic stress (15, 20), and glucose availability (15). A major environmental stress encountered by bacteria in vivo is severe iron restriction; to investigate the role of iron in the regulation of biofilm formation, *S. aureus* strains Newman (7) and 8325-4 (18) and clinical strains CAPD-83, CAPD-84, and CAPD-92 (University Hospitals of Leicester) and B1003003 and B1703012 (Queen's Medical Centre, Nottingham, United Kingdom) were grown in CRPMI medium in 5% CO_2 in air (18) for 24 h, diluted to an optical density at 595 nm (OD_{595}) of 0.1 in fresh CRPMI with and without the addition of 50 μM $Fe_2(SO_4)_3$, and aliquoted into quadruplicate wells of 96-well flat-bottomed tissue culture plates (Nunc). The plates were incubated for 24 h, and biofilm formation was assessed as described previously (6). All strains showed enhanced biofilm formation under low-iron conditions, whereas addition of iron resulted in a significant decrease in biofilm formation (Fig. 1A and B). All strains besides Newman produced low levels of biofilm when iron was depleted, although the enhancement of biofilm formation in comparison to that in iron-replete medium was significant. Slight differences in growth of the different strains in CRPMI were observed, but these were not significant. The effect of iron on biofilm formation is concentration dependent (Fig. 1B); it occurs in other iron-restricted media and is not due to changes in pH resulting from addition of ferric sulfate to the medium, nor is it due to growth in 5% CO_2 in air compared to growth in air (data not shown). Moreover, inhibition of biofilm formation is specific for iron; addition of other metal ions such as magnesium, manganese, copper, and calcium did not consistently affect biofilm formation to any significant degree (data not shown). Thus biofilm formation, an important virulence factor of *S. aureus*, is induced in low-iron growth conditions and repressed by iron.

Previous studies have shown that osmotic stress induces *S. aureus* biofilm formation and that the alternative transcription factor σ^B is involved in this induction (20). *S. aureus* Newman and 8325-4, representing extremes of biofilm-forming ability in low-iron CRPMI, and the isogenic Newman Δ*sigB*::*erm* mutant (10) were used to investigate the combined effect of low iron and osmotic stress on the induction of biofilms and the role of σ^B in these conditions. Strains were grown in iron-restricted and iron-replete CRPMI in the presence and absence of 3% NaCl for 24 h. As shown in Fig. 2A, low iron and high osmotic stress resulted in equally high levels of biofilm formation in Newman and 8325-4, which was comparable to the amount of biofilm produced by Newman in low iron and low osmotic stress. Interestingly, the presence of iron did not completely repress biofilm formation in high osmotic conditions as it does in low osmotic conditions, suggesting that different factors are induced in response to low iron and osmotic stress and that some of these are not repressed by iron. In addition, there were no significant differences in the patterns of biofilm formation between Newman and the Newman Δ*sigB*::*erm* mutant (Fig. 2A) or between 8325-4, which is σ^B deficient, and the σ^B-positive isogenic strain SH1000 (13; data not shown), suggesting that σ^B does not have a significant role in the regulation of biofilm formation in low-iron conditions.

The polymeric *N*-acetylglucosamine polysaccharide (PNAG) (16) encoded by the *ica* locus is proposed to be critical for biofilm formation (4). To determine the effect of low iron and osmotic stress on PNAG production, PNAG levels in cell extracts and culture supernatants were measured directly using a polysaccharide slot blot assay (1) with polyclonal rabbit antiserum specific for PNAG (16). Surprisingly, there was no reproducible significant increase in the level of PNAG extracted from strain Newman grown in low-iron conditions compared to cells grown in iron-replete conditions (as measured by densitometry of repeat blots from individual experiments) to account for the significant difference between Newman biofilm formation in iron-restricted and iron-replete growth conditions

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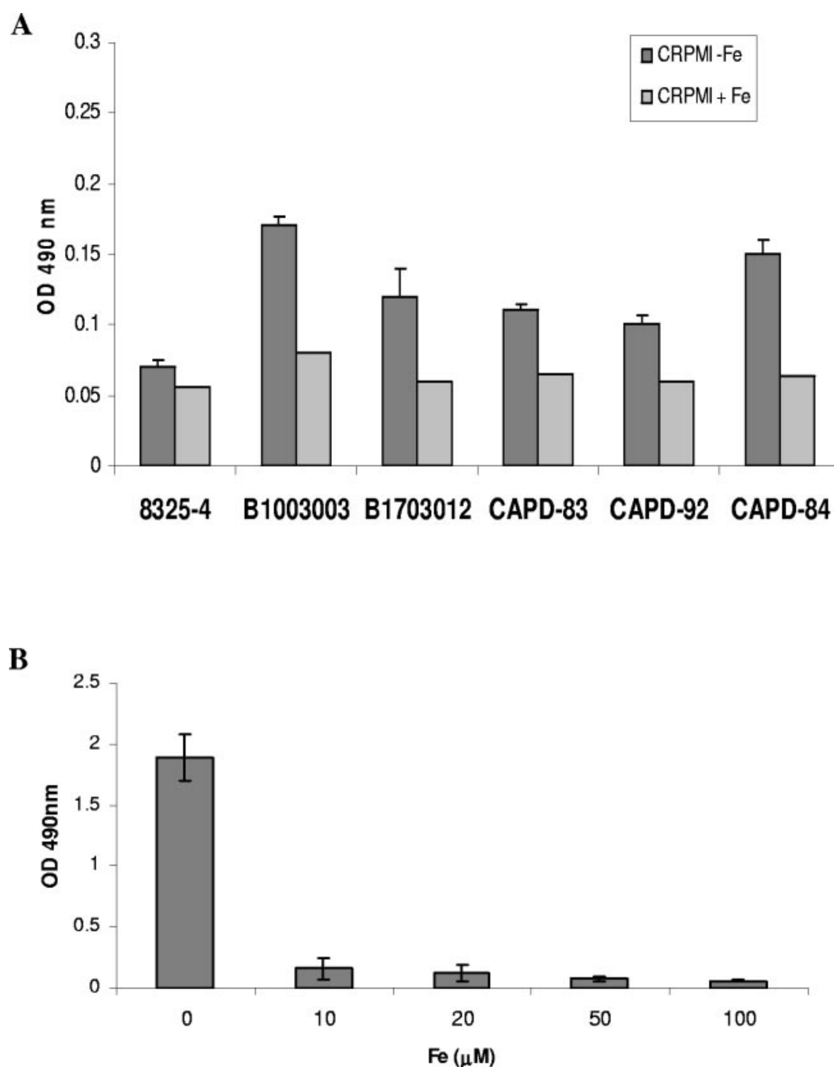

FIG. 1. *S. aureus* biofilm formation is regulated by iron. *S. aureus* strains 8325-4, B1003003, B1703012, CAPD-84, CAPD-83, and CAPD-92 (A) and Newman (B) were grown in CRPMI overnight, diluted to an OD_{595} of 0.1 in CRPMI supplemented with $Fe_2(SO_4)_3$ either at 50 μM (A) or at the concentrations indicated (B), and inoculated into quadruplicate wells of a polystyrene microtiter plate. After 24 h, the wells were washed with phosphate-buffered saline, adherent bacteria were stained with 1% (wt/vol) safranin, and biofilm formation was assessed by OD_{490} measurements. Results represent the averages of at least three independent experiments and the standard error of the mean.

(Fig. 2B). Furthermore, 8325-4 produced a level of PNAG similar to that produced by Newman, and yet 8325-4, in contrast to Newman, produces only a very low level of biofilm in CRPMI (Fig. 2B), and there was no significant increase in PNAG levels in either strain to account for the observed increase in biofilm under osmotic stress conditions. Additionally, although some PNAG was detected in the supernatants, there was again no difference in the relative levels compared to the cell surface extracts that would account for the differences in biofilm levels (data not shown). Thus, these results suggest that biofilm factors other than PNAG are induced in response to low iron and osmotic stress in vitro. These are relevant environmental conditions in vivo, and therefore this observation may explain why a number of studies have shown that *ica* mutants are not attenuated in experimental device-related infection models (8, 9, 14). Therefore, factors other than PNAG may be critical for biofilm formation in vivo.

Iron-responsive gene regulation in *S. aureus* is mediated by the global regulator Fur (12, 21). To determine the role of Fur in iron regulation of biofilm formation, *S. aureus* Newman and the isogenic Newman Δ*fur*::*tet* mutant, constructed by transducing the *fur*::*tet* mutation from 8325-4 (12) with phage ϕ11 (3), were assayed for biofilm formation in iron-restricted and iron-replete CRPMI. If Fur were involved in iron-dependent repression of biofilm production, we would expect to see equal levels of biofilm regardless of iron status. However, biofilm levels were still significantly repressed in the presence of iron in the Newman *fur* mutant ($P = 0.046$), suggesting that Fur is not responsible for the negative iron regulation of *S. aureus* biofilm formation (Fig. 3A). Nevertheless, Fur does appear to

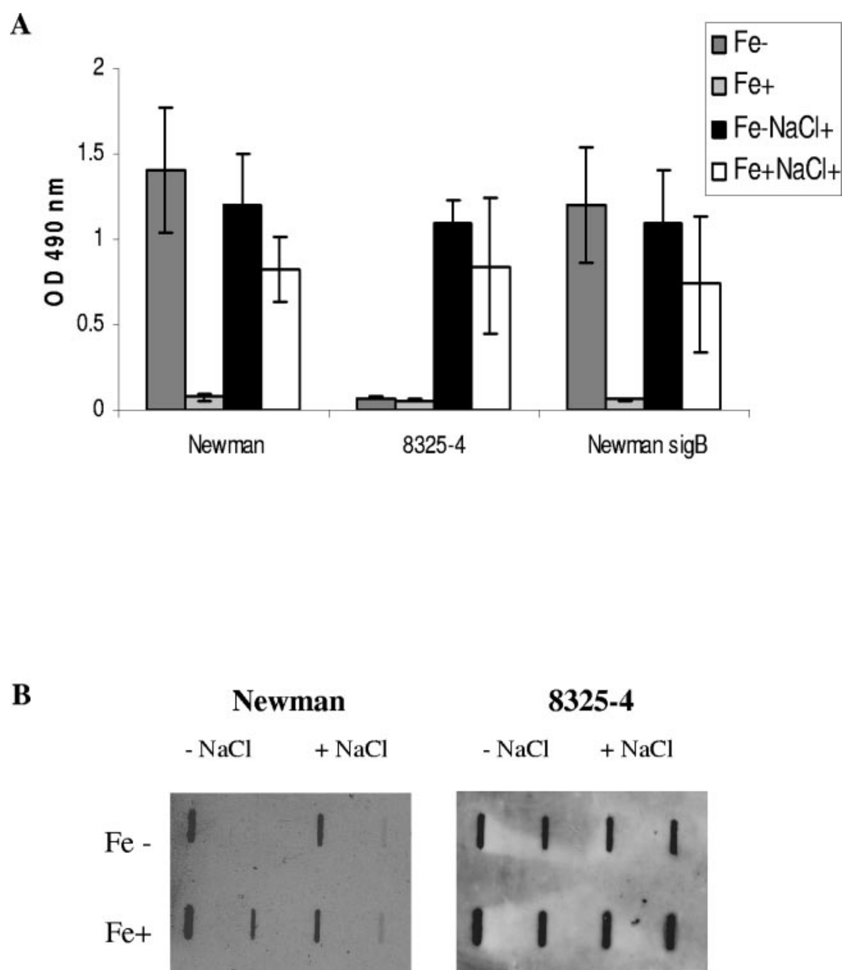

FIG. 2. Biofilm assays and slot blot analysis of PNAG extracts indicate that PNAG levels do not correspond with maximal biofilm levels in *S. aureus*. (A) Biofilm formation by *S. aureus* Newman, 8325-4, and the Newman Δ*sigB*::*erm* mutant. (B) PNAG production by strains Newman and 8325-4. Bacteria were grown in CRPMI overnight and diluted to an OD_{595} of 0.1 in CRPMI (Fe−) or CRPMI plus 50 μM $Fe_2(SO_4)_3$ (Fe+) with (NaCl+) or without the addition of 3% (vol/vol) NaCl and inoculated in quadruplicate into wells of a polystyrene microtiter plate. After 24 h the wells were washed with phosphate-buffered saline, adherent bacteria were stained with safranin, and biofilm formation was assessed by OD_{490} measurements. Results represent the averages of at least eight independent experiments and the standard error of the mean. For measurement of PNAG production, cells were harvested by centrifugation from 24-h static cultures. PNAG was extracted by boiling cell suspensions in EDTA; cell debris was removed by centrifugation, and 50-μl samples of serial dilutions (1:10 and 1:100) were applied to nitrocellulose membrane for probing with PNAG-specific rabbit antiserum. Each experiment was repeated at least three times using PNAG extracts obtained from cultures grown on different days with similar results in each case.

have a significant role in biofilm regulation in Newman; the fourfold decrease ($P = 0.039$) in the level of biofilm produced by the *fur* mutant strain compared to that of wild-type Newman suggests a positive regulatory role in low-iron conditions. This observed decrease was not due to any significant difference in growth between the wild-type and *fur* mutant strains (data not shown). PNAG levels of the *S. aureus* Newman Δ*fur*::*tet* mutant were also measured, but no significant difference was observed in any of the growth conditions or in comparison to the wild type, indicating that Fur does not regulate expression of PNAG (Fig. 3B).

The observation that Fur does not repress *S. aureus* biofilm formation was surprising, as our previous studies indicated that Fur may have a negative regulatory role in initial adhesion (19). Therefore, we investigated the effect of Fur and iron on the earlier stages of *S. aureus* biofilm formation. At 6 h, as at 24 h, biofilm formation by wild-type *S. aureus* Newman showed Fur-independent negative regulation by iron (Fig. 3C). In contrast to positive regulation by Fur at the later stages, however, it appears that Fur negatively regulates the early stages of *S. aureus* biofilm formation in low-iron conditions, as there is a significant increase in biofilm formation at 6 h in the *fur* mutant ($P = 0.008$) (Fig. 3C). There were no significant differences in the growth of the wild-type and mutant strains at 6 h. Thus, Fur both positively and negatively regulates different stages of biofilm formation in low iron. To our knowledge, there have been no reports of Fur acting as an activator protein in low iron in any other bacteria. The mechanisms of regulation in *S. aureus* are unknown: Fur may act indirectly on another regulatory mechanism such as a small RNA (17), or it may repress gene

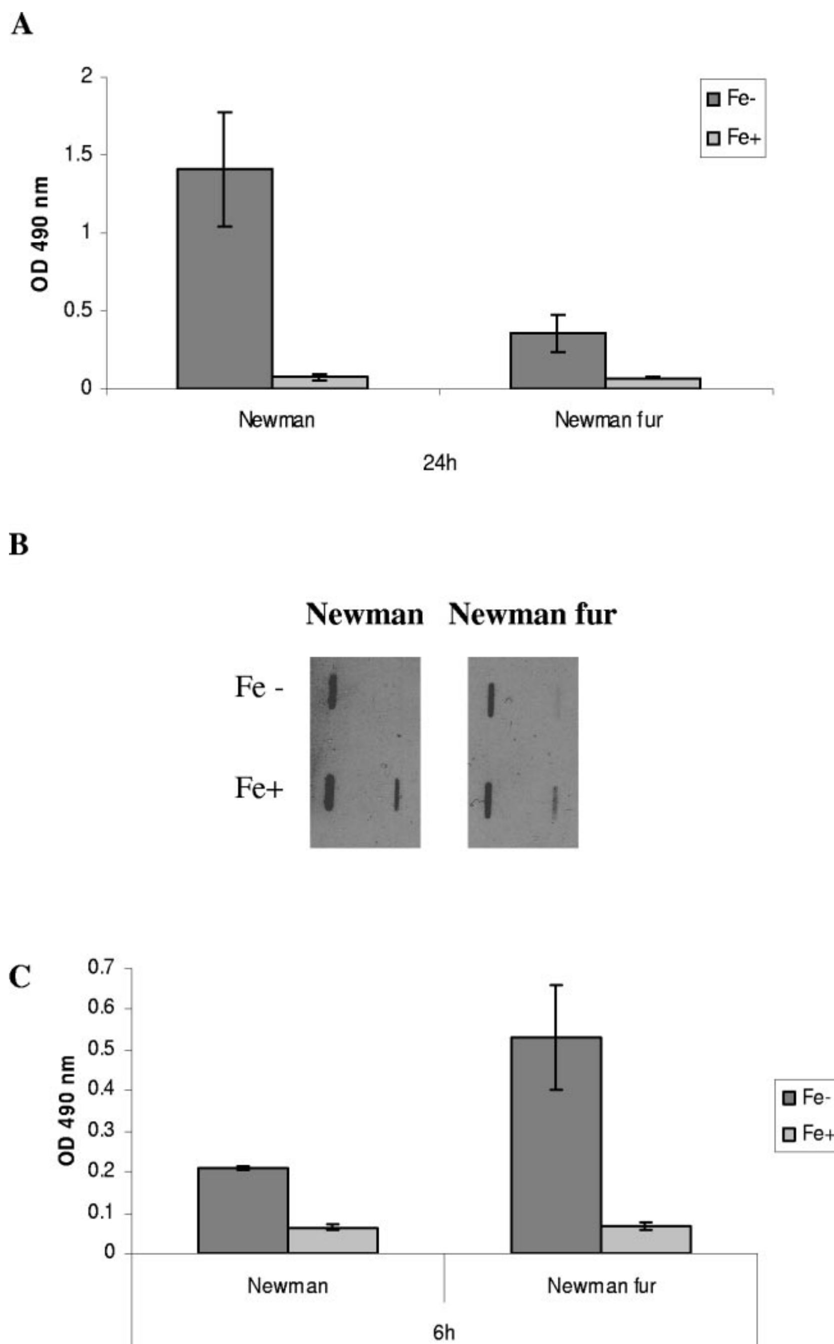

FIG. 3. Effect of *fur* mutation on *S. aureus* biofilm formation. Strain Newman and the isogenic Newman Δ*fur*::*tet* mutant were grown in CRPMI overnight, diluted to an OD_{595} of 0.1 in CRPMI (Fe−) or CRPMI plus 50 μM $Fe_2(SO_4)_3$ (Fe+), and inoculated in quadruplicate into wells of a polystyrene microtiter plate. After (C) 6 h or (A) 24 h, the wells were washed with phosphate-buffered saline, adherent bacteria were stained with safranin, and OD_{490} measurements were obtained. Results represent the averages of at least eight independent experiments and the standard error of the mean. For measurement of PNAG production (B), cells were harvested by centrifugation from 24-h static cultures. Fifty-microliter samples of serial dilutions of PNAG extracts (1:10 and 1:100) were applied to nitrocellulose membrane for probing with PNAG-specific rabbit antiserum. Each experiment was repeated at least three times using PNAG extracts obtained from cultures grown on different days with similar results in each case.

expression by directly binding promoters in low iron as observed in *Helicobacter pylori* (2).

In summary, we have demonstrated that iron-mediated repression of biofilm production is Fur independent and that Fur has both positive and negative regulatory roles in low iron. We have also demonstrated that factors other than PNAG are critical for biofilms induced in low-iron growth conditions; there is significant strain variation in the expression of these factors, suggesting that regulation of *S. aureus* biofilm formation is extremely complex.

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